Integrated karyotyping of sorghum by in situ hybridization of landed BACs

Jeong-Soon Kim, Kevin L. Childs, M. Nurul Islam-Faridi, Monica A. Menz, Robert R. Klein, Patricia E. Klein, H. James Price, John E. Mullet, and David M. Stelly

Abstract: The reliability of genome analysis and proficiency of genetic manipulation are increased by assignment of linkage groups to specific chromosomes, placement of centromeres, and orientation with respect to telomeres. We have endeavored to establish means to enable these steps in sorghum (*Sorghum bicolor* (L.) Moench), the genome of which contains ca. 780 Mbp spread across $n = 10$ chromosomes. Our approach relies on fluorescence in situ hybridization (FISH) and integrated structural genomic resources, including large-insert genomic clones in bacterial artificial chromosome (BAC) libraries. To develop robust FISH probes, we selected sorghum BACs by association with molecular markers that map near the ends of linkage groups, in regions inferred to be high in recombination. Overall, we selected 22 BACs that encompass the 10 linkage groups. As a prelude to development of a multiprobe FISH cocktail, we evaluated BAC-derived probes individually and in small groups. Biotin- and digoxygenin-labeled probes were made directly from the BAC clones and hybridized in situ to chromosomes *without* using suppressive unlabelled C_0t-1 DNA. Based on FISH-signal strength and the relative degree of background signal, we judged 19 BAC-derived probes to be satisfactory. Based on their relative position, and collective association with all 10 linkage groups, we chose 17 of the 19 BACs to develop a 17-locus probe cocktail for dual-color detection. FISH of the cocktail allowed *simultaneous* identification of all 10 chromosomes. The results indicate that linkage and physical maps of sorghum allow facile selection of BAC clones according to position and FISH-signal quality. This capability will enable development of a high-quality molecular cytogenetic map and an integrated genomics system for sorghum, without need of chromosome flow sorting or microdissection. Moreover, transgeneric FISH experiments suggest that the sorghum system might be applicable to other Gramineae.

Key words: integrated karyotyping, FISH, sorghum, BAC.

Résumé : La fiabilité de l'analyse génomique et les possibilités de modification génétique se trouvent accrues par l'assignation de groupes de liaison à des chromosomes spécifiques, par la localisation des centromères et par la détermination de l'orientation relative aux télomères. Les auteurs ont cherché à développer les moyens techniques nécessaires à l'atteinte de ces objectifs chez le sorgho (*Sorghum bicolor* (L.) Moench), une espèce dont le génome compte environ 780 Mb distribués sur $n = 10$ chromosomes. L'approche repose sur l'emploi de l'hybridation in situ en fluorescence (FISH) et de ressources intégrées en génomique structurale dont des banques de chromosomes bactériens artificiels (BAC) à inserts de grande taille. Afin de produire des sondes FISH robustes, les auteurs ont sélectionné des clones BAC portant des marqueurs moléculaires situés près des extrémités des groupes de liaison, dans des régions postulées comme étant riches en événements de recombinaison. En tout, les auteurs ont choisi 22 clones BAC qui couvrent les 10 groupes de liaison. Préalablement au développement d'une sonde FISH à plusieurs composantes, les sondes dérivées de BAC ont été évaluées individuellement et en petits groupes. Des sondes marquées à la biotine ou à la dioxygénine ont été produites directement à partir des clones BAC et hybridées in situ aux chromosomes sans utiliser d'ADN C_0t-1 non-marqué comme agent de blocage. En fonction de l'intensité du signal FISH et du degré relatif de bruit de fond, 19 sondes ont été jugées satisfaisantes. En tenant compte de leur localisation et de leur représentation des 10 groupes de liaison, 17 des 19 BAC ont été employés afin de mettre au point une sonde mixte (à 17 composantes) pour des fins de détection en mode bicolore. Une hybridation FISH avec la sonde mixte a permis d'identifier simultanément tous les 10 chromosomes. Les résultats indiquent que les cartes génétiques et physiques du

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sorgho permettent une sélection aisée des clones BAC en fonction de leur position et de la qualité de leur signal FISH. Cette capacité permettra de développer une carte cytogénétique moléculaire de grande qualité et un cadre intégré d'analyse génomique pour le sorgho sans avoir besoin de recourir à la sélection chromosomique en flux ou à la microdissection. De plus, des expériences d'hybridation FISH intergénérique suggèrent que le système développé chez le sorgho pourrait s'avérer utile chez d'autres hordées.

Mots clés : caryotypage intégré, FISH, sorgho, BAC.

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Introduction

Reliable cytological techniques for chromosome identification are necessary for efficient genome research and germplasm utilization. The discoveries of mitogens, hypotonic bursting, and chromosome banding that led to facile methods of mitotic-cell recovery, chromosome spreading, and segmental identification collectively revolutionized the cytogenetics of humans and many other animals (Hsu 1979). Physical mapping of repeated sequences, genomic clones, and cDNAs based on fluorescence in situ hybridization (FISH) has been an important feature of human genomics. Chromosomal and subchromosomal localization of breakpoints and in situ hybridized probes relied on a backdrop of routinely produced karyotypes composed of 400+ bands (Lichter et al. 1990; Gingrich et al. 1993; Moir et al. 1994; Muleris et al. 1994). More recently, close to 9000 bacterial artrificial chromosomes (BACs) of the Human Genome Project have been "FISHed" to confirm their integrity and point of origin, cross-check critical framework maps, reveal duplications and paralogy, and provide an integrated resource for genomics and gene identification, for example, positional candidate gene identification for traits with known cytogenetic aberrations (Cheung et al. 2001; http://www.ncbi.nlm.nih.gov/genome/cyto/hbrc.shtml). These operations underscore the feasibility of using large-insert libraries and integrated genomics resources for development of molecular cytogenetic resources.

Chromosome-banding methods have, unfortunately, been far less effective in most plants than in mammals and have severely limited the utility of conventional karyotypic analysis in the development of plant genomics. Efficient karyotypic methods to screen for segmental chromosomal variation are still lacking in all but a few species with very large chromosomes (e.g., wheat $2n = 42$, ca. 16 000 Mbp/1C). Innovative approaches offer useful alternatives in some species (e.g., maize–oat addition line radiation hybrids (Riera-Lizarazu et al. 2000) and wheat gametocidal system-induced segmental deletion lines (Endo and Gill 1996)) but solutions offering general applicability are desirable. In most plant species, a detailed cytogenetic framework that would otherwise expedite the development and integration of genomics resources is still lacking. The need for such capabilities in plant genomics is acute, owing to the prevalence of polyploidy among plants. For example, the rate among angiosperms is in the range of 40–70% (Stebbins 1950; Masterson 1994). Recent evidence of polyploid features in angiosperms heretofore widely regarded as diploid, for example, maize (Moore et al. 1995) and *Arabidopsis thaliana* (Blanc et al. 2000; The Arabidopsis Initiative 2000), suggests that rates of polyploidy among angiosperms are higher than previously suspected. The dosage and organizational changes resulting from polyploidy, segmental duplications, deletions, and rearrangements are of significant concern, owing to their effects on genome mapping, clonal-library coverage, screening efficacy, contig assembly, and the uniqueness of genes and gene products. The importance of karyotypic systems and integrative genome mapping is thus especially important in plant genomics and genome manipulation.

Sorghum (*Sorghum bicolor* (L.) Moench, $2n = 20$) is a domesticated grass grown in many tropical and subtropical regions. Its importance is accentuated in many agriculturally recalcitrant semi-arid regions, for example, in Africa. Yu et al. (1991) identified every chromosome of the sorghum cultivar Combine Kafir 60, using a Giemsa C-banding technique in conjunction with chromosome length and arm-ratio measurements. While the work of Yu et al. (1991) was a significant step forward, contemporary genomics research and breeding efforts require the development of integrated genome analysis tools with a resolution greater than C-banding can provide. Moreover, cytogenetic variability across germplasm might compromise the applicability of C-band-based karyotypes. In theory, FISH signals could serve as excellent cytological markers for chromosome or segment identification based on locus-specific FISH signals. Thus, we endeavor to develop a FISH-based karyotyping system for sorghum and other gramineous species. Herein, we report the results of one of the first steps—tagging each of the 10 sorghum chromosome pairs in a karyotypically distinct manner.

There are essentially two strategies for FISH-based karyotyping. The simpler one is based on patterns from one or more multilocus repeated sequences that yield FISH signal at a limited number of discrete loci, as exemplified by multicolor FISH of two or three tandemized repetitive sequences in spruce (Brown and Carlson 1997; Brown et al. 1998; http://dendrome.ucdavis.edu/Image/kary2.html). Tandemly arranged repeated sequences, such as rRNA genes, that are at one or a few loci in the genome can serve as robust FISH-based karyotypic markers. However, the rarity of such loci limits their utility—in sorghum there is just one large 18S–5.8S–26S rRNA gene cluster (Sang and Liang 2000) and just one moderately sized 5S rRNA gene cluster (M.N. Islam-Faridi, unpublished results). A second strategy for FISH-based karyotyping could be based on the collective use of multiple low-copy sequences, each of which hybridizes to just one location per genome, or perhaps to a few, owing to duplications and (or) polyploidy. Low-copy clones are potentially a much more plentiful source of probes, especially given the rapid development of large-insert libraries and related genomic infrastructure in many taxonomic groups. To achieve robust FISH signals from low-copy sites, mid-sized

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Fig. 1. BACs identified by screening the BAC pools with AFLP markers were individually checked for the presence of those markers. The four sorghum BACs sbb6585, sbb9251, sbb17392, and sbb24713 were identified when the BAC pools were screened using primers that amplified AFLP markers *Xtxa224* (LG-A), *Xtxa516* (LG-D), *Xtxa391* (LG-B), and *Xtxa567* (LG-F), respectively. Each of these BACs was isolated, prepared as AFLP template, and used in an AFLP reaction with the appropriate fluorescently labeled AFLP primers (*Xtxa224*, E-CAA + M-CTG; *Xtxa516*, E-TGA + M-CCT; *Xtxa391*, E-TGA + M-CGA; *Xtxa567*, E-TGA + M-CGC). AFLP-template DNA from the mapping parents (IS3620C and BTx623) were also used in the same AFLP reactions. Fluorescently labeled products were run on a LI-COR DNA sequencer. The figure shows the gel images from these AFLP reactions. Marker names are given adjacent to the bands that are polymorphic between the mapping parents and that were specifically amplified from the individual BACs. Molecular-weight markers are shown in lane M. All reactions were performed according to the method of Klein et al. (2000).

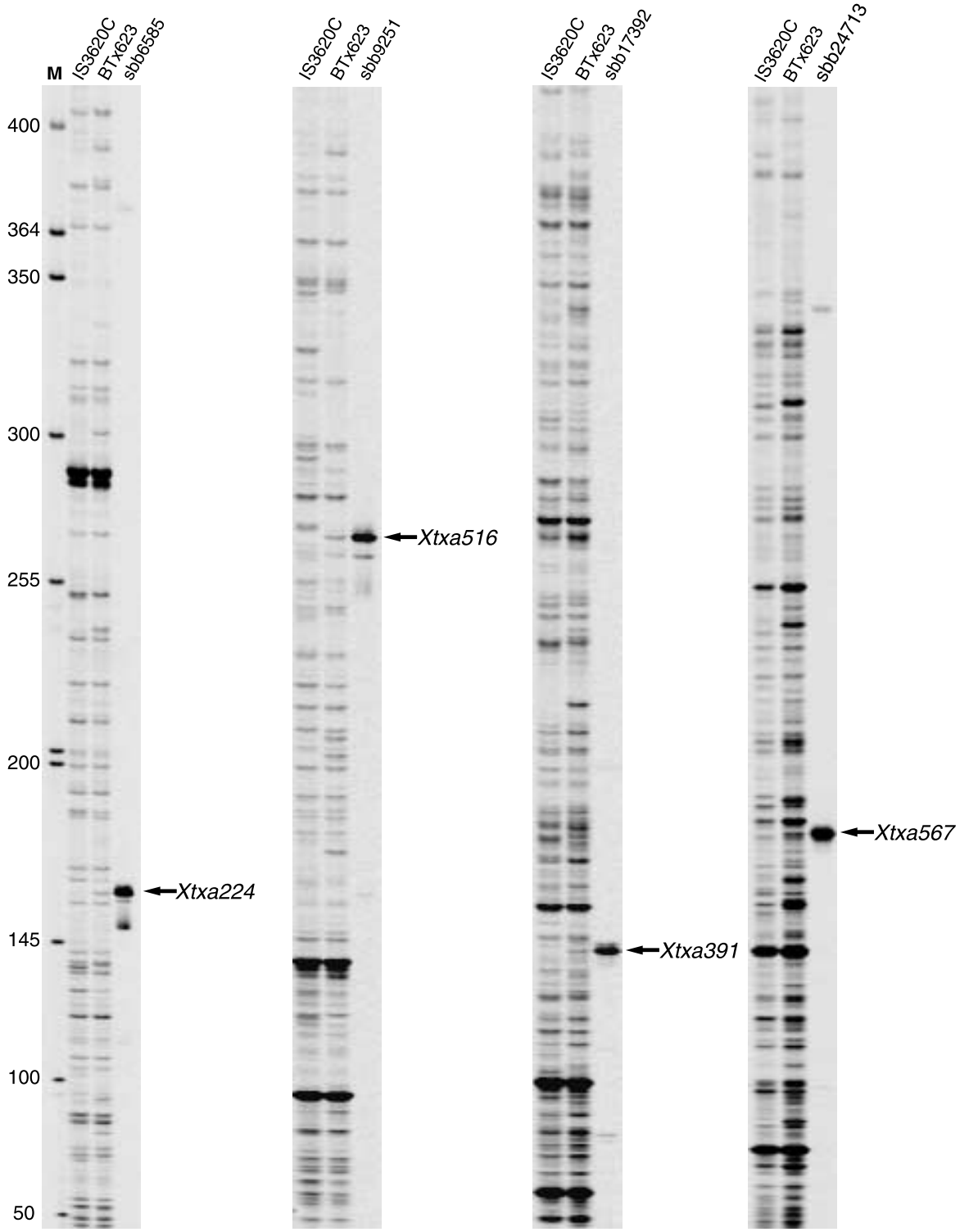

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Fig. 2. BACs tested for repetitive sequences on the sorghum chromosomes without the aid of C_0t-1 DNA for blocking. Figures A–J show hybridization from one BAC probe from each of the 10 different linkage groups. Arrowheads indicate pairs of BAC-FISH signals. Scale bar = 5 μm.

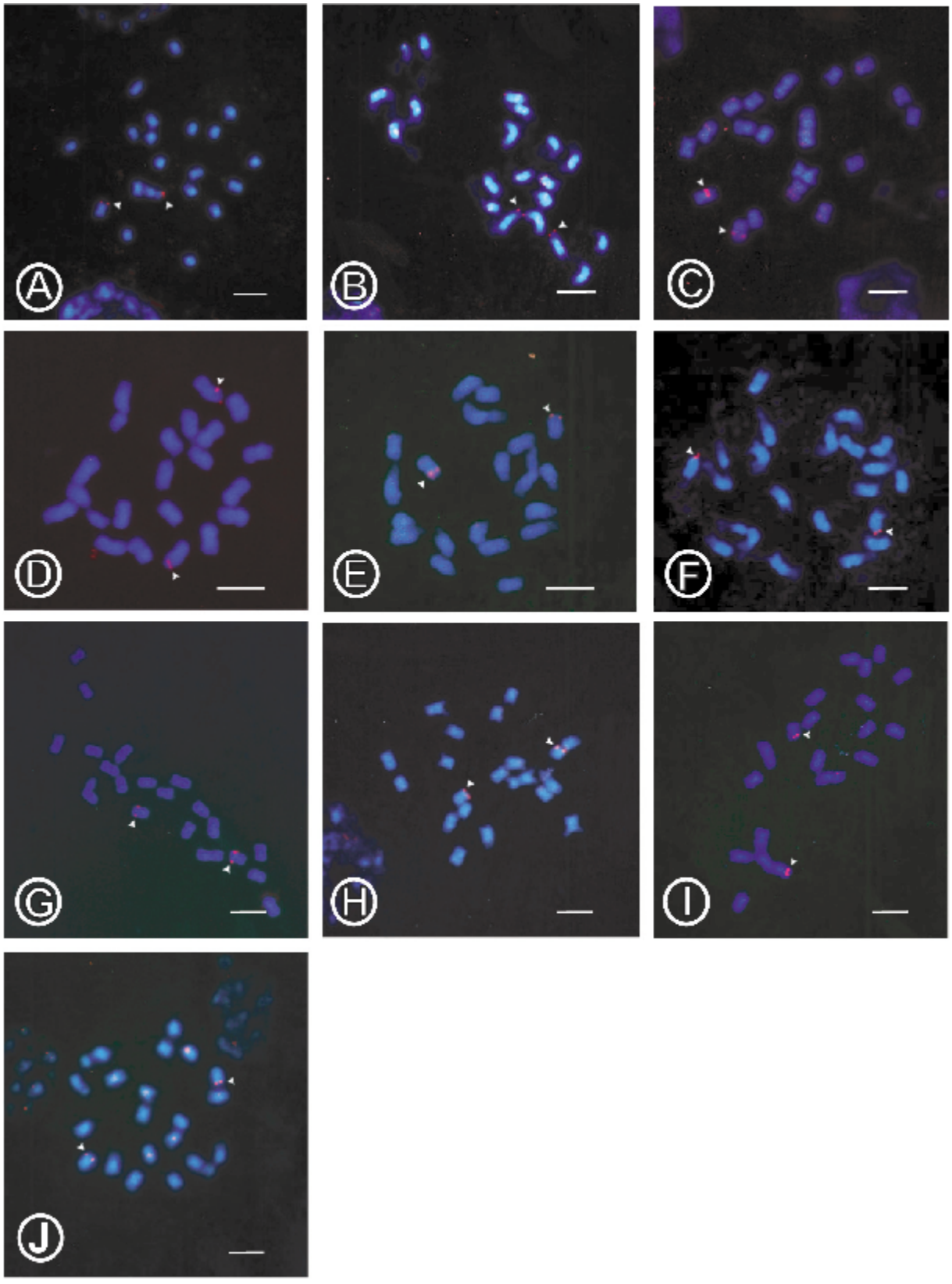

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Fig. 3. (A–D) Two-color FISH to test synteny of BACs associated with loci from sorghum linkage groups. Scale bar = 5 μm. (A) Linkage group I. (B) Linkage group B. (C) Linkage group C. (D) Linkage group H. (E–H) Single (DAPI or FITC), dual (DAPI, Cy3), and triple (DAPI, FITC, Cy3) bandpass filter images from a single chromosome spread after simultaneous hybridization of 17 sorghum BACs, following single and combinatorial labeling of individual BACs with biotin- and digoxigenin-haptens, respectively, detected with FITC (green) and Cy3 (red). Pairs of letters denote the respective linkage group of the sorghum map (Menz et al. 2002). Filled arrowheads denote BAC probes labeled with both biotin and digoxigenin (1:1). Scale bar = 5 μm. (E–G) Photographs of fresh preparations. (E) DAPI-counterstained sorghum chromosomes. (F) Cy3 image showing hybridization signals of eight digoxigenin-labeled BACs and two BACs labeled with both haptens (filled arrowhead). (G) FITC image showing pairs of primary hybridization signals for seven biotin-labeled BACs, one pair of secondary signals for sbb23575 (open arrowhead), and two BACs labeled with both haptens (filled arrowhead). (H) Digital image from a 3-week-old FISH preparation showing hybridization of 17 sorghum BACs. The yellowish color colocalized with strong Cy3 red signals was artifactual, owing to the high level digital enhancement used to accentuate the faded FITC signal.

(cosmid) and large-insert DNA clones, for example, BACs (Lichter et al. 1990; Hori et al. 1992; Takahashi et al. 1992; Hanson et al. 1995; Fillon et al. 1998; Dong et al. 2000; Song et al. 2000), can be used as cytological markers to tag individual chromosomes. Many large-insert genomic clones that contain low-copy sequences also contain repetitive sequences that hamper or preclude detection of the low-copy sequences by FISH. When homologous repetitive sequences are abundant in the target genome and the BAC probe, BAC FISH often results in a FISH signal that is widely distributed across the genome. Such a signal creates "noise" that essentially camouflages signals occurring at the BAC-homologous sequence locus. While "blocking" with unlabeled C_0t-1 or other repetitive fractions can alleviate moderate noise, it is insufficient in some instances. Moreover, the process is protected by patent (U.S. Patent No. 5,447,841).

FISH of most plant BACs commonly results in excessive "noise" from multi-site hybridization of repeated sequences. Competitive in situ suppression with blocking DNA, usually unlabeled C_0t-1 fraction genomic DNA, helps significantly for many BACs. For other BACs, however, excess blocking DNA cannot adequately preclude hybridization by labeled probe that contains repeated sequences, so background hybridization patterns remain significant. Thus, when discrete FISH loci are sought, it is helpful to select clones containing relatively large amounts of unique sequences and relatively small amounts of dispersed repetitive sequences. Similar considerations guided the production of region-specific FISH probes from flow-sorted chromosomes, microdissected chromatin, and other large-insert clones, such as yeast artificial chromosomes (YACs).

In sorghum, regions adjacent to centromeres are largely heterochromatic and regions near the ends of chromosomes are euchromatic. In previous work on sorghum, most BACs yielding discrete FISH signals originated from the ends of chromosome arms, that is, from euchromatic regions (Gomez 1997; Zwick et al. 1998). As one of the steps in development of a facile molecular cytogenetic system for sorghum, we devised and tested strategies that would expedite the development of BAC probes amenable to FISH, that is, "FISHable." To identify FISHable BACs, we selected BACs containing markers located near the ends of each of the individual linkage groups. Here we report the selection of BAC probes that can be used for FISH. The probes provide an excellent means of identifying sorghum chromosomes, initially integrating structural and functional genomics resources with chromosomal features, and initiating development of a robust molecular cytogenetic system for sorghum and other gramineous species.

Materials and methods

Selection of BACs for FISH

The BACs used in this study came from two genomic BAC libraries that were derived from the sorghum cultivar BTx623, and collectively contain 26 016 clones with an average insert size of ca. 150 kbp, that is, about 5× sorghum genome equivalents (Woo et al. 1994; Tao and Zhang 1998). A "sbb" (*S. bicolor* BAC) number refers to a BAC from these libraries. The BACs from the Woo et al. (1994) library are numbered sbb1–sbb13440, and the BACs from the Tao and Zhang (1998) library are numbered sbb13441–sbb26016.

Molecular markers from a high-density genetic map of sorghum were used to screen a complex set of BAC pools (Klein et al. 2000; Menz et al. 2002). To increase the likelihood that syntenic BAC FISH loci would be distinguishable from each other after hybridization to chromosomes, the markers from a common linkage group were selected only if separated by a substantial genetic distance. Additionally, only markers that mapped to regions of apparently high recombination near the ends of linkage groups were considered for use in this study. For BACs selected by AFLP (amplified fragment length polymorphism) or simple sequence repeat (SSR) markers, the primers for the appropriate AFLP or SSR were used for screening the BAC pools by PCR. For BACs selected by restriction fragment length polymorphism (RFLP) markers, the clone for the RFLP was sequenced, and specific primers were designed for the RFLP clone. The RFLP-specific primers were then used to screen the BAC pools by PCR. In all cases, BACs initially identified by the results from BAC-pool screening were individually rescreened using PCR primers for the marker that identified that BAC. Most of the BACs used as FISH probes were identified by direct association with mapped markers. However, five of the BACs used as probes (sbb19188, sbb10186, sbb10491, sbb10718, and sbb12906) were indirectly identified by mapped markers as members of fingerprinted BAC contigs that were identified by a mapped marker. BAC sbb3766 was used because it contains the phytochrome *PHYA* gene and has been fully sequenced (Genbank accession AF369906; D.T. Morishige, K.L. Childs, J.E. Mullet, unpublished data).

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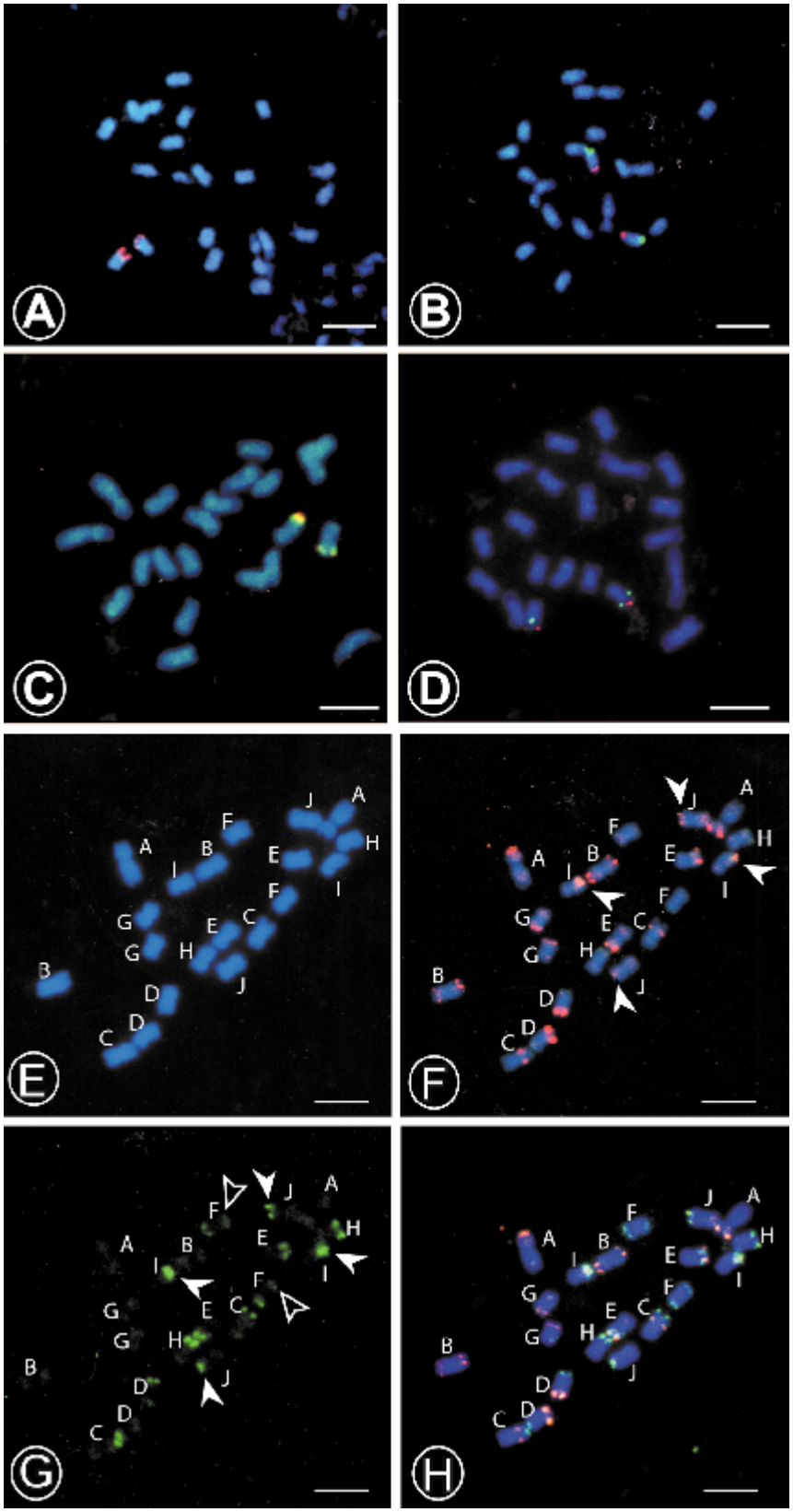

Metaphase preparation

Sorghum (*S. bicolor* (L.) Moench) plants (BTx623) were grown under glasshouse conditions. Excised root tips were treated with saturated aqueous α-monobromonaphthalene solution at room temperature for 1.5 h, fixed in ethanol – acetic acid (3:1), and rinsed in water for 10 min. Excised meristematic tips were placed in enzyme solution (5% cellulase and 2.5% pectolyase in 0.1 M citrate buffer) at

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Fig. 4. Comparisons of linkage map (A), chromosome diagram (B), and FISH images (C, D, and E) for linkage groups A–J. Scale bar = 50 cM on the linkage maps. (A) Linkage maps showing a partial set of loci from each linkage group, including the markers used to select BACs for FISH (Menz et al. 2002). (B) Diagrams of chromosomes in which the colored shapes represent signal of BAC clones selected by markers on A. Green square indicates FITC detection; red circle, Cy3 detection; pink triangle, combined Cy3 + FITC detection. (C–E) Paired images of multi-site, multi-BAC FISH experiment. (C) Digital image from 3-week-old FISH preparation showing DAPI, Cy3, and FITC signals. Signal distributions correspond to individually photographed Cy3 (D) and FITC (E) images. In the digital image, the yellowish color colocalized with strong Cy3 red signals was artifactual, owing to the high level of digital enhancement used to accentuate the faded FITC signal (green channel). (D and E) Photographs of fresh preparations. (D) DAPI + Cy3 image taken photographically. (E) FITC image taken photographically. Arrowhead shown for linkage group F indicates the minor signal of BAC clone sbb23575 of linkage group H.

37°C for 40 min, rinsed with distilled water, placed on a clean glass slide with a drop of ethanol – acetic acid (3:1), macerated using fine-pointed forceps, allowed to air-dry at room temperature for 2 days, and stored in a freezer at –20°C.

BAC DNA purification and probe labeling

BAC DNA was isolated by alkaline lysis, digested with *Eco*RI, and then further purified using Plant DNeasy spin columns (Qiagen, Valencia, Calif.), using a modified protocol (Childs et al. 2001). The purified BAC DNA was labeled with biotin-16-dUTP or digoxigenin-11-dUTP by the BioNick Labeling system (Roche Molecular Biochemicals, Indianapolis, Ind., U.S.A.).

In situ hybridization

In situ hybridization techniques were a modification of Jewell and Islam-Faridi (1994), as described by Hanson et al. (1996). Slides were immersed in 30 µg RNase/mL 2× SSC (1× SSC: 0.15 M NaCl plus 0.015 M sodium citrate) for 45 min at 37°C and then washed in 2× SSC. The chromosomal DNA on the glass slide was then denatured at 70°C in 70% formamide in 2× SSC for 1.5 min, followed by dehydration in 70, 85, 95, and 100% ethanol, for 2 min each, at –20°C. The hybridization mixture (25 µL for one slide) contained 10 ng of labeled probe DNA, 50% formamide, and 10% dextran sulfate in 2× SSC. The mixture was denatured at 90°C for 10 min, chilled on ice, and added to the slide. Following overnight incubation at 37°C, slides were rinsed at 40°C in 2× SSC, 50% formamide in 2× SSC, 2× SSC, and 4× SSC plus 0.2% Tween 20, for 5 min each. Slides were blocked 5 min at room temperature with 5% (w/v) BSA in 4× SSC plus 0.2% Tween 20. Biotin-labeled probes were detected with 1% Cy3™-conjugated streptavidin and digoxygenin-labeled probes with 1% fluorescein isothiocyanate (FITC)-conjugated anti-digoxygenin antibody. Slides were washed 3 times in 4× SSC plus 0.2% Tween 20 for 5 min at 37°C. DAPI (4′,6-diamidino-2-phenylindole) in McIlvaine's buffer (9 mM citric acid, 80 mM $Na_2HPO_4 \cdot H_2O$, 2.5 mM $MgCl_2$ (pH 7.0)) was used for counterstaining chromosomes. After rinsing, Vectashield antifade solution (Vector Laboratories, Inc.) was applied.

Microscopy

Images were viewed through an Olympus AX-70 epifluorescence microscope equipped with standard filter cubes. Prior to acquiring a digital imaging system, images were recorded photographically using Fuji HG ASA 400 professional film. Later, images from a Peltier-cooled 1.3 M pixel Sensys camera (Roper Scientific) were captured with the MacProbe v.4.2.3 digital image system (Applied Imaging Corp., Santa Clara, Calif., U.S.A.).

Results

Isolation of chromosome-specific BAC clones of sorghum

Marker-based BAC screening is generally assumed to be error free, but errors could arise from infidelity of methods, erroneous data handling, or redundancies within genomes, that is, repeated sequences, segmental duplications, or polyploidy. To minimize the possibility of such errors in this study, all BACs identified by screening the BAC DNA pools with mapped markers were determined to truly contain the mapped markers by rescreening the individual BAC DNAs (Fig. 1). In all cases tested, the expected markers were clearly present in the selected BACs.

BAC-FISH signal strength and distribution

Twenty-two sorghum BACs that were placed on the 10 linkage groups of a high-density sorghum genetic map (Menz et al. 2002) were selected according to map position and tested by FISH for repetitive sequences. FISH of probes from individual BACs indicated that 19 of the 22 clones produced little or no background signal when hybridized to sorghum chromosomes *without* the aid of C_0t-1 DNA for blocking (Fig. 2). Only three clones resulted in moderate background signal when hybridized without blocking DNA (data not shown). One of the three BACs, sbb3766, has been fully sequenced and is known to contain five retrotransposonlike elements, along with 18 genes (Genbank accession AF369906; D.T. Morishige, K.L. Childs, J.E. Mullet, unpublished data).

BAC-FISH signal synteny

To develop a pilot karyotyping system for the simultaneous identification of all sorghum chromosomes, we needed to develop distribution–color "signatures" of individual or pairs of syntenic BAC-FISH probes that would be diagnostic for each chromosome. To test the expected synteny of BACs selected with markers from a common linkage group, we used two-color detection of FISH for complementarily labeled pairs and sets of probes. Each pair or set of BACs associated with linked marker loci yielded FISH signals on a common chromosome pair (Figs. 3A–3D).

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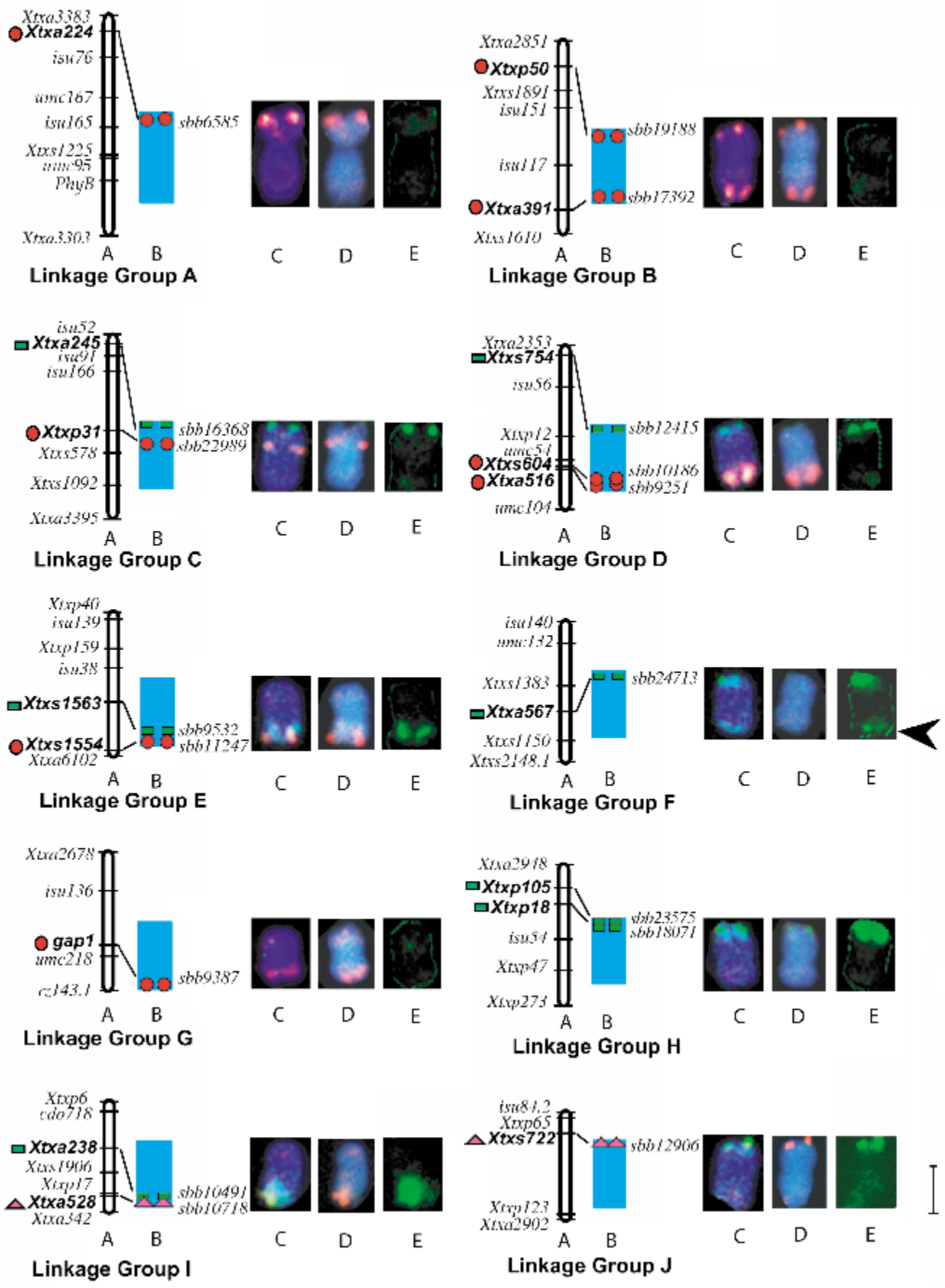

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Locations of secondary signals

When FISH results in signals at more than one site, it is important to determine if there is a primary hybridization site, which is usually larger and brighter than the signal at nonprimary sites. It is also important to determine that secondary signal sites are constant and represented in both chromatids, since this indicates that they arose from hybridization based on homology rather than from nonspecific binding of the probe or fluorochrome conjugate. A few of the BACs in this experiment yielded FISH signals at more than one locus. The secondary signals were usually detected on both sister chromatids of both homologues, which discounted origins not dependent on homology.

In contrast with the primary hybridization sites, for which the chromosome identity is known through the linkage group association of the BAC marker, the identity of the chromosome with which such secondary sites are associated is not implicitly known, unless it happens to be syntenic with the primary BAC-FISH site. Using FISH-based karyotyping, we were able to observe secondary signals on chromosomes other than those bearing the main signal. For example, the secondary signal of sbb23575, which contains linkage group H marker *Xtxp105*, was found on the chromosome bearing the primary signal of sbb24713, which contains linkage group F marker txa567 (Fig. 4).

Simultaneous chromosome identification

Based on the above results, probes made from 17 clones distributed across the 10 linkage groups were pooled in a BAC-FISH cocktail and used to simultaneously identify all 10 chromosome pairs in two-color FISH on mitotic chromosomes. In the resulting preparations, each chromosome had one, two, or three different BAC-FISH loci with various color(s) of signal (Figs. 3E–3H). Using the distribution of pattern and color of signals, all chromosome pairs were readily identified. The relative positions of FISH markers enabled comparisons of physical positions of markers in the chromosomes and linkage maps (Fig. 4). Although several of the BACs (*Xtxp31*, *Xtxs1563*, *gap1*, *Xtxa238*, and *Xtxa567*) were associated with linkage markers that defined interior positions within their respective linkage groups, all the FISH markers except sbb22989 (*Xtxp31*) were located near the ends of chromosomes.

Discussion

The facility of large-insert library construction and high-quality large-insert clonal DNA isolation renders them highly amenable to targeted and large-scale development of FISH probes. In this study, we targeted each of the 10 chromosomes of *S. bicolor* for development of one or more high quality FISH probes that would associate each chromosome with one of the 10 linkage groups (Chittenden et al. 1994; Kong et al. 2000; Menz et al. 2002). Furthermore, we required probes that could be FISHed while using only modest or, preferably, no "blocking" with unlabelled repetitive DNA sequences (for example, C_0t-1 fraction equivalents), because the sensitivity of FISH is highest when blocking DNA is not used.

Most BAC libraries are composed of genomic clones that are 100–200 kb in size, and the targets of FISH are thus relatively large and easy to detect. Such large genomic clones are, however, more likely than small clones to contain dispersed repetitive sequences that cause high levels of FISH background signal. With the aim of simultaneously localizing many BACs on individual chromosome spreads, we sought to pick BACs with relatively low repetitive sequence content and relatively high gene content, or at least high unique sequence content. The avoidance of repetitive elements can be critical to the success of BAC FISH. For example, sequence analysis revealed that BAC sbb3766 contains only five regions of repetitive sequence (D.T. Morishige, K.L. Childs, J.E. Mullet, unpublished data). Nevertheless, when this BAC was tested using FISH without suppressive DNA, it produced a painting-like signal over all the chromosomes. This indicates that even a small amount of certain repetitive sequences in a BAC can result in significant background signal. In previous studies, we have often used Southern blots of individual BAC DNAs probed with labeled genomic DNA to screen against BACs with abundant repetitive sequences (e.g., Hanson et al. 1995). Here, our approach was indirect. We selected BACs that were identified by molecular markers that mapped to regions of high recombination near the ends of each linkage group of the sorghum linkage map (Menz et al. 2002). Several previous and recent studies have shown that recombination rates are correlated to several features, including high GC content, high gene density, and a relatively low density of at least certain repetitive sequences (Collins et al. 1996; Gill et al. 1996; Barakat et al. 1997; Broman et al. 1998). We anticipated that BAC clonal inserts originating from these regions of the genetic map would be rich in low copy sequence content and lacking in repetitive-sequence content. Thus, FISH probes made directly from these BACs were expected to require little or no suppressive hybridization to achieve unambiguous FISH-based localization. Several recent studies have extended the correlation among GC content, gene density, recombination, and other features (Barakat et al. 1999; Faris et al. 2000; Gerton et al. 2000; McCombie et al. 2000). Recent work in sorghum using cDNA selection with sorghum BACs has also shown that BACs linked to markers from regions of high recombination tend to be gene rich (Childs et al. 2001), lending support to this simple strategy.

Some BACs, for example, sbb6585, yield one or more pairs of secondary hybridization signals that are less intense than the primary-signal sites. The mapping of secondary signals is crucial to molecular cytogenetic applications and also to detection of redundancies within genomes. When such secondary FISH sites are syntenic with the respective primary FISH site, the chromosomal location is implicit. However, additional information is needed when secondary FISH sites are nonsyntenic with the primary FISH sites. The ability to identify the chromosomal location of secondary BAC-FISH sites is not assured unless the spread is karyotyped. For example, the secondary signal of sbb23575 (linkage group H) was nonsyntenic to its primary signal but was syntenic to sbb24713 signal (linkage group F). The ability to identify each of the chromosomes across multiple cells by FISH-based karyotyping is expected to facilitate the analysis of the sorghum and related genomes for segmental duplicated segments, polyploidy, and repeated-sequence distributions.

During the development of linkage maps, the numbers of

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markers is typically less than desired and the degree of genomic coverage is uncertain. The degree to which a linkage map provides good coverage can be grossly evaluated by FISH of BACs selected with markers from the ends of linkage groups, as exemplified in bovine by FISH of microsatellite-selected BACs (De Donato et al. 1999). In sorghum, FISH of BACs selected with markers from the ends of linkage groups B and D yielded FISH signals located at the ends of the respective chromosome arms. The findings indicate that the physical coverage of the chromosomes is nearly complete for linkage groups B and D. The two markers Xtxa238 and Xtxa528 define a segment that spans over 50% of linkage group I in the BTx623 × IS3620C sorghum linkage map (Menz et al. 2002), whereas their respective BAC-FISH signals were very near each other on the distal region of the same arm. This reinforces the concept that the recombinationally active regions of sorghum chromosomes are primarily in the large distal euchromatic segments.

The results indicate that linkage and physical maps of sorghum can be used to facilitate development of an integrated map that includes a chromosomal map of the sorghum genome. The feasibility of identifying sorghum BACs that are highly amenable to simultaneous FISH and FISH-based karyotyping is clearly demonstrated for segments that are gene rich and high in recombination. Approaches that take advantage of large-insert libraries and integrated genomic resources seem largely to obviate the need of flow sorting or microdissection for FISH-probe development. Complementary large-insert libraries based on different restriction digests offer excellent coverage (Tao et al. 2001), are amenable to large-scale robotic screening and manipulation, and allow for facile high-quality DNA extraction prior to probe development. In terms of FISH-probe development, approaches based on integrated structural genomic resources will allow for targeting, precision, and breadth. The approach exemplified herein indicates that development of an extensive molecular cytogenetic karyotyping system for sorghum is highly feasible. When linked directly to linkage and physical (contig) maps, BAC-FISH markers assume greater importance, as an integrative genomics resource from which plant research will profit markedly. Moreover, we have shown previously that transgeneric marker-based BAC selection and transgeneric-BAC FISH are quite feasible among at least some Gramineae, for example, rice, sorghum, sugarcane, and maize (Zwick et al. 1998). We expect, therefore, that probes from a comprehensive molecular system for sorghum are likely to be applicable to other gramineous species and, thus, constitute a framework for development of a molecular cytogenetic system for Gramineae. Such a system would facilitate both integrative and comparative genomics among gramineous species.

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